

Selected Zingiberaceae Species Exhibiting Inhibitory Activity Against *Mycobacterium tuberculosis* H₃₇Rv: A Phytochemical Profile

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Abstract

As part of the research efforts to identify plant species which may have potential against tuberculosis, a study was earlier conducted in collaboration with the Institute for TB Research, University of Illinois, Chicago, to randomly screen the crude alcoholic extracts of different plant species using the MABA assay, to determine any inhibitory activity against the causative agent, *Mycobacterium tuberculosis* H₃₇Rv. Of the five species belonging to the family Zingiberaceae, four were found to inhibit the growth of *M. tuberculosis* H₃₇Rv. These species included *Alpinia purpurata* (Vieill.) K. Schum., *Alpinia zerumbet* (Pers.) B.L.Burtt. & R.M. Sm., *Etingera elatior* (Jack) R.M. Sm. and *Zingiber officinale* Roscoe. Each species was collected in bulk and subjected to extraction and several bioassay-directed chromatographic fractionations. The pure constituents obtained were analyzed for their structure using spectroscopic techniques. The bioactivity of the pure isolates, as minimum inhibitory concentration values, was likewise determined. The results showed the antitubercular activity to be present in the nonpolar extracts. Structure elucidation of the pure isolates revealed the presence of sterols (β -sitosterol, stigmasterol), sterol derivatives (β -sitosteryl- β -D-galactoside, β -sitosteryl-3-O-6'-palmityl- β -D-glucoside), phenyldecanoids (6-shogaol and 6-gingerol) and a flavonoid (kumatakenin). Determination of the MIC showed higher activity of the phenyldecanoids than the steroids, the steroid derivatives and the flavonoid.

Introduction

Tuberculosis is a pandemic that has been afflicting the Philippines and other developing countries. The Philippines is one of six countries with half of all new cases. The number of reported new TB cases keeps rising. Because of

the rapid increase in TB incidence in Africa, there is a yearly 1% growing incidence worldwide. Data show that one third of the world's population is infected with *M. tuberculosis* and every year, nearly 2 million deaths are caused by TB (WHO, 2006a, b).

A number of risk factors affecting TB are HIV/AIDS and multiple drug resistance. For people living with HIV/AIDS, TB is the single biggest killer. Latent TB infection is reactivated to an active disease through HIV. The other risk factor, multiple drug resistance, leads to TB that does not respond to the standard drug treatment. A WHO survey indicated the presence of MDR-TB in 109 countries, with the highest rates in China and former Soviet Union. In 2006, WHO launched a six component "Stop TB Strategy." (WHO, 2006b).

Tuberculosis is caused by *Mycobacterium tuberculosis* (M tb), an intracellular pathogen affecting higher vertebrates. The search for drugs against tuberculosis uses the slow growing M tb in its bioassays. In the late 1980's, the UST Research Center for the Natural Sciences (RCNS) embarked on a natural products program to screen plants using *Mycobacterium* 607 or *M. smegmatis*, a surrogate fast growing and non-pathogenic organism. After a number of comparative tests using both organisms failed to show 100% agreement, it was deemed necessary to screen against the actual etiologic agent, *M. tuberculosis*, a unique organism with unique susceptibilities to drugs.

The Philippines is one of those countries in Southeast Asia with a rich diversity of flora. Though only 858 species were reported to be medicinal (Quisumbing, 1978), there are many more plants whose medicinal properties have not been tested. The general objective of the RCNS TB Group is to provide a scientific rationalization for the use of plants as sources of medicine against TB, either for phytopharmaceutical application or for new drug development. Its specific objectives include the random screening of plants for inhibitory activity against *M. tuberculosis* (done in collaboration with the Institute for TB Research of the University of Illinois in Chicago), identifying plant families / genera exhibiting inhibitory activities, isolating through a bioassay-guided procedure the constituents in the active fractions, identifying the structure of the constituents through a combination of spectroscopic methods, comparing structural characteristics and determining the minimum inhibitory concentration of the pure isolate. This paper collates the results of the different studies done on five species of Zingiberaceae, namely, *Alpinia purpurata* (Vieill.) K. Schum., *Alpinia zerumbet* (Pers.) B.L.Burtt. & R.M. Sm., *Etlingera elatior* (Jack) R.M. Sm., *Hedychium coronarium* J. König and *Zingiber officinale* Roscoe (Aguinaldo *et al.*, 1997; Agbayani *et al.*, 2002; Budoy *et al.*, 2004; Villaflores *et al.*, 2004; Villaflores, 2005).

Materials and Methods

Plant material

The voucher specimens were identified and kept at the UST Herbarium or the National Museum. Table 1 lists the herbarium vouchers, including locality, collection number, and collector.

Table 1. Herbarium voucher information (location, collection date & number, collector, herbarium identity).

	Location	Date of Collection	Collection number	Collector	Where deposited
<i>Alpinia purpurata</i>	Los Banos, Laguna	Feb 2004	USTH 4717	O. Villaflorres	UST Herbarium
<i>Etlingera elatior</i>	Los Banos, Laguna	Apr 2002	Ref. No. 2002 - 120	C. Budoy	National Museum (PNH)
<i>Alpinia zerumbet</i>	Los Banos, Laguna	Apr 2002	Ref. No. 2002 - 113	M. Agbayani V. Arbolante	National Museum (PNH)
<i>Zingiber officinale</i>	Tuguegarao, Cagayan	Dec 2001	Ref. No. 2000 - 0481	M. Agbayani	National Museum (PNH)
<i>Hedychium coronarium</i>	Tuguegarao, Cagayan	Dec 2001	Ref. No. 2000 - 0481	M. Agbayani	National Museum (PNH)

Screening for bioactivity

Approximately 100g plant material (air-dried leaves, fresh rhizomes, fresh flowers) was ground and extracted with methanol (or ethanol). The filtrate was concentrated in vacuo at 40°C to give a crude extract. A portion (2 mg) of the crude extract was tested for % inhibitory activity against *M. tuberculosis* H₃₇Rv (Collins and Franzblau, 1997; Fischer *et al.*, 1998). If the crude extract showed activity, it was partitioned between water and hexane, dichloromethane and 1-butanol. Each organic layer was concentrated and a portion similarly tested for bioactivity.

Isolation (bioassay-guided) and structure elucidation

The plant materials (air-dried leaves of *A. purpurata*, fresh rhizomes of *Z. officinale* and *E. elatior*) were collected in bulk and extracted exhaustively with alcohol. The crude extract obtained from each was partitioned as above to obtain the active semicrude extract. The latter was fractionated repeatedly using silica gel column chromatography with gradient elution (hexane-DCM, DCM-MeOH) till pure isolates were obtained. Fractions

from each chromatographic step were assayed for bioactivity. Isolates were analyzed using a combination of spectroscopic techniques such as UV, IR, MS, ¹H-NMR and ¹³C-NMR, including DEPT, COSY, HMQC and HMBC. These were again assayed for minimum inhibitory concentration (MIC).

Results and Discussion

Table 2 shows the percent inhibitory activity of the crude extracts against *M. tuberculosis* H₃₇Rv at 100 ug/mL. *A. purpurata* leaves exhibited the highest activity (94%, 100 ug/mL), followed by *E. elatior* rhizomes (86%, 100 ug/mL), and *A. zerumbet* (80%, 100 ug/mL). *Z. officinale* showed activity in the rhizomes but absence of activity in the leaves. Among the five species of Zingiberaceae tested, only *H. coronarium* did not exhibit any activity against *M. tuberculosis* H₃₇Rv. Since only the leaves of *H. coronarium* were tested and not the rhizomes, it is worth considering the rhizomes for future tests.

Table 2. Activity of the crude extracts vs. *M. tuberculosis* H₃₇Rv at 100 ug/mL.

	Inhibition (%) - rhizomes	Inhibition (%) - leaves
<i>Alpinia purpurata</i>	Not tested	94
<i>Etlingera elatior</i>	86	38
<i>Alpinia zerumbet</i>	80	Not tested
<i>Zingiber officinale</i>	41	-35
<i>Hedychium coronarium</i>	-59	Not tested

When the crude extracts of *A. purpurata* rhizomes, leaves and flowers were tested, the leaves showed the highest activity at two concentrations (62%, 64 ug/mL; 41%, 32 ug/mL) (Table 3). The percent inhibition values of the rhizomes and flowers were close (34% and 30%, 64 ug/mL; 21% and 17%, 32 ug/mL) and indicated less activity than the leaves.

Table 3. Activity of the crude extracts from the different plant parts of *A. purpurata* vs. *M. tuberculosis* H₃₇Rv.

Plant part	Inhibition (%) at 64 ug/mL	Inhibition (%) at 32 ug/mL	Inhibition (%) at 16 ug/mL
Rhizomes	34	21	12
Leaves	62	41	4
Flowers	30	17	7

Upon separation of the nonpolar, semipolar and polar constituents in the crude leaf extract of *A. purpurata*, the higher activity is in both hexane and DCM semicrude extracts (64-70%, 64 ug/mL; 58-61%, 32 ug/mL; 34-42%, 16 ug/mL) (Table 4). This indicates that the bioactive constituents have a nonpolar character.

Table 4. Activity of the semi-crude extracts from *A. purpurata* leaves vs. *M. tuberculosis* H³⁷Rv.

	Inhibition (%) at 64 ug/mL	Inhibition (%) at 32 ug/mL	Inhibition (%) at 16 ug/mL
Hexane	64	61	34
DCM	70	58	42
n-BuOH	35	10	6

For *Z. officinale* (Table 5), the bioactive constituents are largely nonpolar, being present in the hexane semicrude extract (61%, 100 ug/mL; 19%, 25 ug/mL), with an activity much higher than that in the DCM (9%, 100 ug/mL) or n-BuOH (-10%, 100ug/mL). Table 5 shows that for the rhizomes of *E. elatior*, the active constituents are in the nonpolar (hexane) and semipolar (DCM) extracts (34% and 35%, respectively, 100 ug/mL). The data for *A. zerumbet* show an activity for the DCM extract (76%, 50 ug/mL) which is more than triple that of the hexane extract (23%, 50 ug/mL). Tables 4 and 5 compare activities of the semicrude extracts per plant in order to determine the presence of the active constituents. The different studies used varied concentrations of the semicrude extracts.

Table 5. Activity of the semi-crude extracts from *Z. officinale* rhizomes, *E. elatior* rhizomes and *A. zerumbet* rhizomes vs. *M. tuberculosis* H³⁷Rv.

	<i>Z. officinale</i> rhizomes		<i>E. elatior</i> rhizomes	<i>A. zerumbet</i> rhizomes
	Inhibition (%) at 100 ug/mL	Inhibition (%) at 25 ug/mL	Inhibition (%) at 100 ug/mL	Inhibition (%) at 50 ug/mL
Hexane	61	19	34	23
DCM	9	-4	35	76
n-BuOH	-10	-8	0	Not tested

Extracts from the *Z. officinale*, *A. purpurata* and *E. elatior* were subjected to bioassay-guided isolation till pure isolates were obtained (Fig. 1). The details of the isolation, purification and structure elucidation are written elsewhere. From *Z. officinale* rhizomes, the phenyldecanoids 6-

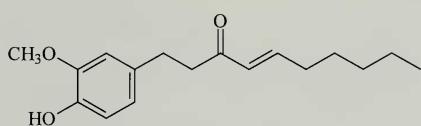
shogaol and 6-gingerol were isolated from the hexane extract. The sterols β -sitosterol and stigmasterol were obtained from the hexane extract of *E. elatior* rhizomes. And from the leaves of *A. purpurata* were obtained β -sitosteryl- β -D-galactoside, β -sitosteryl-3-O-6'-palmityl- β -D-glucoside, and kumatakenin, a flavonoid. The structures were identified upon spectral analysis and comparison of spectral data with the literature (Zaeoung *et al.*, 2005; Wright *et al.*, 1978; Gomes and Alegrio, 1998; Shaiq *et al.*, 2002; Urbatsch *et al.*, 1976; Wang *et al.*, 1989).

Upon bioassay of these isolates, the following MICs were obtained: *Z. officinale*: 6-shogaol (MIC 64 ug.mL), 6-gingerol (MIC 33 ug/mL); *A. purpurata*: β -sitosteryl- β -D-galactoside (MIC >128 ug/mL), β -sitosteryl-3-O-6'-palmityl- β -D-glucoside (MIC >128 ug/mL), kumatakenin (MIC >128 ug/mL); *E. elatior*: stigmasterol (MIC >128 ug/mL), β -sitosterol (MIC >128 ug/mL). These results show the higher activity of the phenyldecanoid isolates from *Z. officinale* than the sterol glycosides, flavonoid from *A. purpurata* and sterols from *E. elatior*. Furthermore, the high percent inhibitory activity values of the crude extracts do not necessarily correlate with those of the pure isolates. With *Z. officinale*, there was an observed increase in activity as purification progressed. This was not observed with *A. purpurata* or *E. elatior* where constituents seem to exhibit synergism and are therefore more active as a mixture.

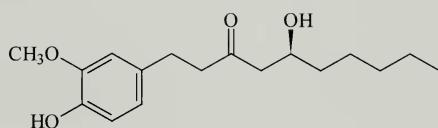
Having observed a common antitubercular property of extracts from Zingiberaceae species randomly selected, it is now worth investigating the other species of Zingiberaceae for reasons of bioactivity targeted search, and probable taxonomic utilization. With the furtherance of investigations on equally or more active species, there is sufficient justification for a comprehensive phytochemical reexamination of natural products elaborated by this family.

Acknowledgements

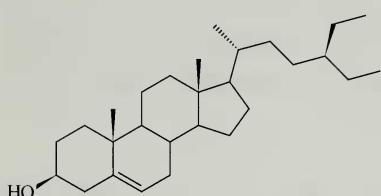
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Figure 1. Compounds isolated from Zingiberaceae species.

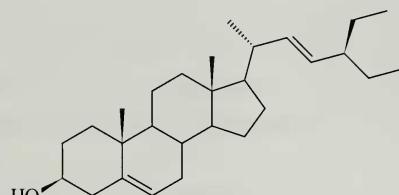
6-shogaol



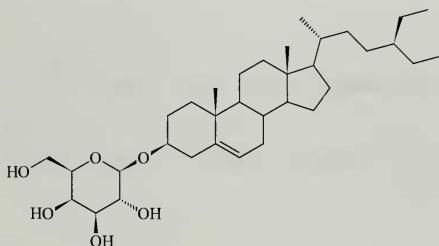
6-gingerol



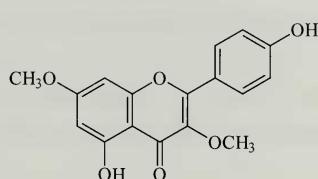
β-sitosterol



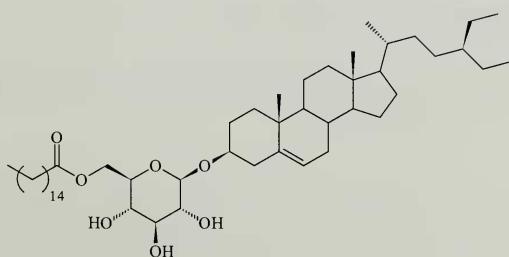
stigmasterol



β-sitosteryl-β-D-galactoside



kumatakenin



β-sitosteryl-3-O-6'-palmityl-β-D-glucoside

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